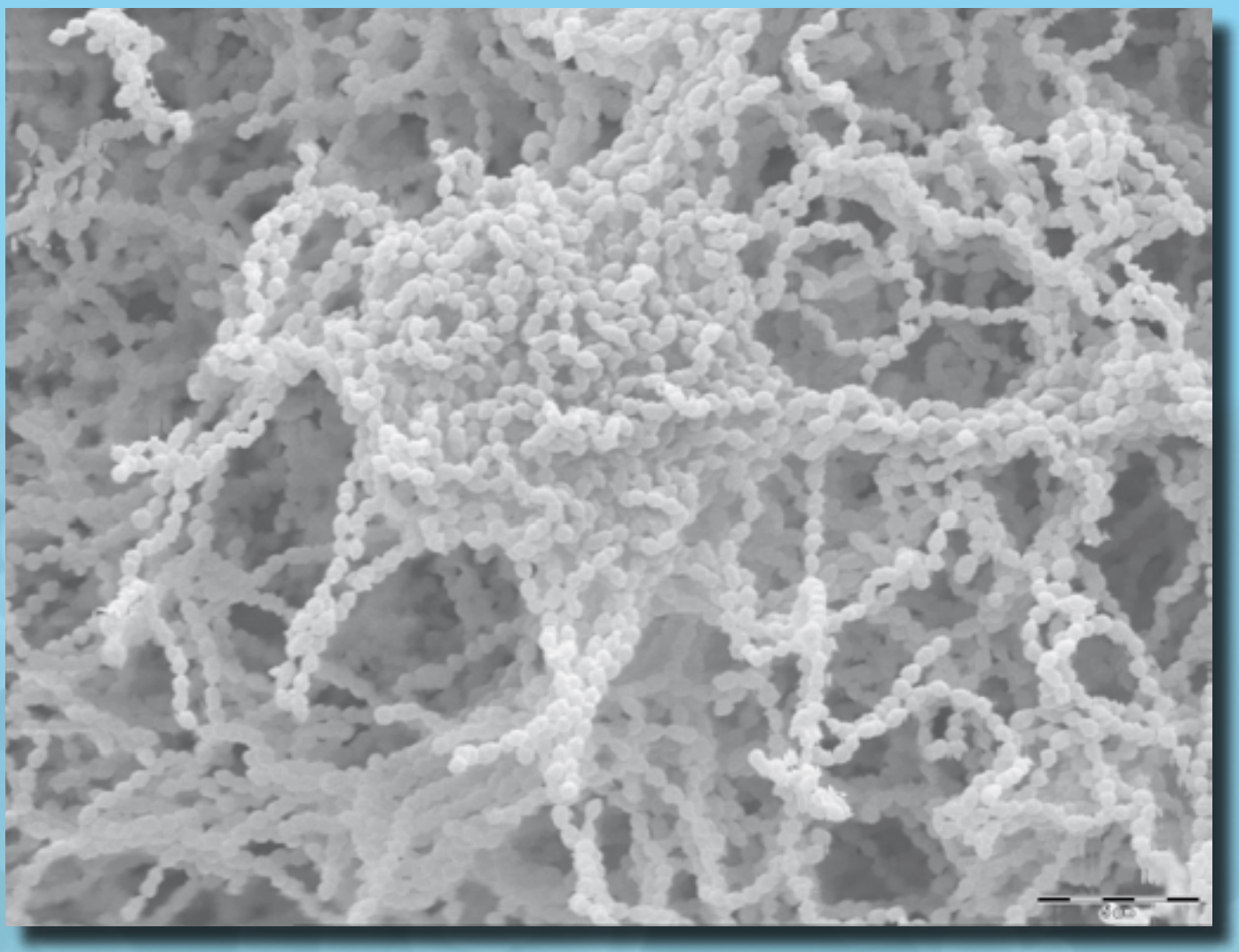
Involvement of *luxS* gene in biofilm formation by *Streptococcus intermedius*



Thesis by Stud. odont Melody Moezzi, Dental Faculty, UiO

Supervisor:
Prof. Dr. odont Anne Aamdal Scheie, Dep. of Oral Biology, UiO.

Co-supervisor:
Post. Doc. Fernanda C. Petersen, Dep. of Oral Biology, UiO

Introduction

Biofilm formation is of great interest to many scientific fields. Not only is the role of biofilm known in their prosperity to coat and corrode pipes for example in the field of petroleum, but their role in modern medicine is of great importance. Approximately 65% of all human bacterial infections such as osteomyelitis, bacterial prostatitis, infective endocarditis, cystic fibrosis pneumonia and oral diseases such as dental caries and periodontal diseases involve biofilms (5).

The biofilm consists of bacteria with a three-dimensional organization, enveloped in extra cellular polymeric substances, and with networks of intervening water channels. The water channels deliver necessary nutrients and remove wastes so that the bacteria deep in the biofilm may survive (4). Bacteria in biofilms are physiologically differentiated from free living bacteria (6).

Until recently, bacteria were considered to represent non- social individual single-cell life forms, lacking the ability to communicate (2). As results from recent studies show, however bacteria are able to organize into groups and they may communicate.

Quorum sensing is a process in which bacteria monitor their cell population density by measuring the concentration of small secreted signal molecules, called autoinducers (3). Quorum sensing requires a sufficient density of bacteria. In biofilms there is a high density of bacteria, which may suggest that communication systems are functional in the biofilms (6). Bacterial communication may be significant in development of many diseases.

Through the process of quorum sensing, bacterial populations coordinate quorum sensing communication systems. The first, known as intraspecies quorum systems, are species specific. The second system is involved in both intra and interspecies communication.

The main communication signal in this system is, called autoinducer 2 (AI-2) (1). The *luxS* gene which is present in many gram negative and gram positive bacteria, is thought to be responsible for synthesizing autoinducer-2 (AI-2) (7,9,10).

A recent study in *Streptococcus gordonii* shows that *luxS* mutation, results in development of a biofilm architecturally different from that of the wild type (9). Another study in *Streptococcus mutans* shows that by the inactivation of *luxS*, the biofilm structure changes, compared to that of the wild type (7). None of these studies show a significant difference in the amount of the biofilm formed by the wild type compared to the mutant (9, 7).

Streptococcus intermedius is an oral bacterium and belongs to the anginosus group. Bacteria in the anginosus group are part of the normal microbiota, found in genitalia, the oropharynx and the intestines. They are often associated with abscesses in internal organs, such as in the brain, lung and liver, as well as local meningitis and pleuraempyema (11).

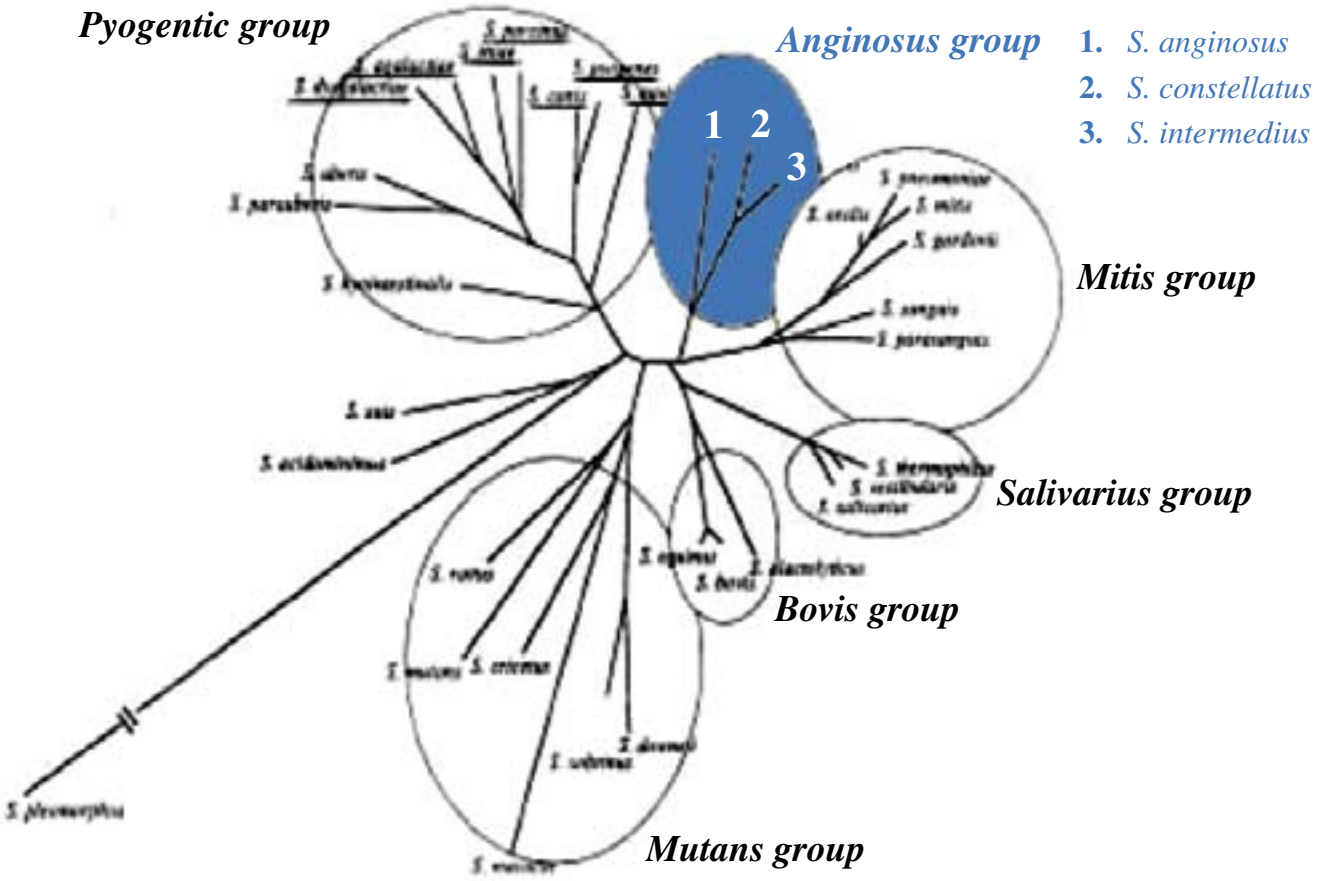


Fig.1
The anginosus group (12).

The role of *luxS* in biofilm formation of *S. intermedius* has not yet been investigated.

Aim

The aim of this study was to investigate whether inactivation of the *luxS* gene was associated with changes in biofilm formation.

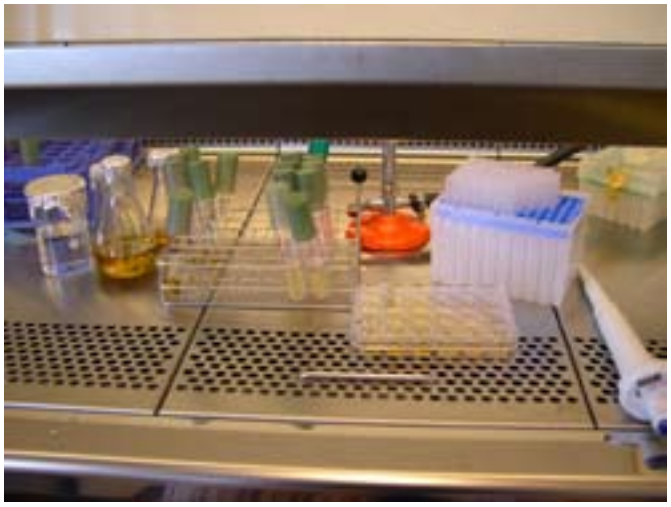
Bacteria and growth media

Biofilm:

S. intermedius wild-type NCTC 11324 and its deletion mutant defective in the *luxS* gene (*luxS÷*) were assayed for their biofilm formation, and then compared.

1. Quantitative method- polystyrene microtiter-plates

The wild-type strain was subcultured on different media. In the first subculture we used Tryptone Soya Broth (TSB). The *luxS÷* were grown using the same medium supplemented with 5µ/ml kanamycin. This would select the mutant only, because in their construction they were rendered kanamycin resistant. To make sure that kanamycin would not interfere with the assay results we used kanamycin only in the first overnight subculture.



We transferred colonies from THB agar plates to liquid medium (TSB) and incubated the cells at 37˚C overnight in a 5% CO_2 aerobic atmosphere. A volume of 50 µl was then transferred into fresh TSB medium the next day, and grown again overnight in the CO_2 incubator. The third day we diluted the second overnight culture in liquid medium (5µl/ml in fresh TSB), and transferred 500 µl into the wells of the microtiter plate. The microtiter plate was incubated at 37˚C overnight in 5% CO_2 aerobic atmosphere. We also gram stained from the second over night batch and studied the specimens under the light microscope. We did this to make sure that both the mutant and the wild type were free from contamination and that they had similar chain formations.

The last day we transferred the planktonic fraction from the wells into Eppendorf tubes and then measured optical density in the spectrophotometer at OD_{600}. The spectrophotometer determines the microbial mass by measurement of light absorption. We did the same with the biofilm fraction. First we added TSB to the wells, scraped off the attached biofilm cells, transferred the resuspended biofilm to Eppendorf tubes. To disrupt coccal chains and to dispense the cells we used the whirlimixer 3×20 sec and then measured OD_{600}.

We also tested biofilm growth of the wild type and *luxS÷* in Brain Heart Infusion medium (BHI). We had three parallels with BHI supplemented with glucose (0, 8%), four parallels with BHI alone, and three parallels with BHI supplemented with sucrose (0,8%).

Growth

We investigated the growth rate of *S. intermedius* wild type and its isogenic mutant. We wanted to seclude any differences in the growth rate between the wild type and the mutant.

The bacteria were cultivated in liquid medium (TSB). In the first over night subculture of *luxS÷* mutant we used kanamycin (5µ/ml).
The second overnight was deluted 1:20 in TSB and incubated at 37ºC in 5 % CO_2 aerobic atmosphere. The wild type and the *luxS÷* mutant growth were monitored by measuring the optical density at 600 nm at different time intervals (fig 2).

Scanning electron microscopy

To study possible structural differences between the *luxS÷* mutant and the wild type biofilm we used scanning electron microscopy. This method allowed us to examine the surfaces of the bacteria in great detail.

Results

Growth curve

When bacteria are introduced into fresh medium, usually no immediate increase in cell number or mass occurs, and therefore this period is called the lag phase.
An obvious lag phase was not observed in the wild type or the *luxS÷* (fig. 2).This may be because measurements were taken only after 2h.
During the exponential or the log phase bacteria are growing and dividing at the maximal rate possible. Because each individual bacterium divides at a slightly different moment, the growth curve rises smoothly rather then in discrete jumps. There was not a significant difference between the mutant and the wild type in this phase.
Eventually the population growth ceases and the growth curve flattens, this is called the stationary phase. In this phase the total number of viable bacteria remains constant until the cells start dying. Microbial populations enter the stationary stage for several reasons.
Obvious reasons are nutrient limitation and accumulation of toxic waste products.

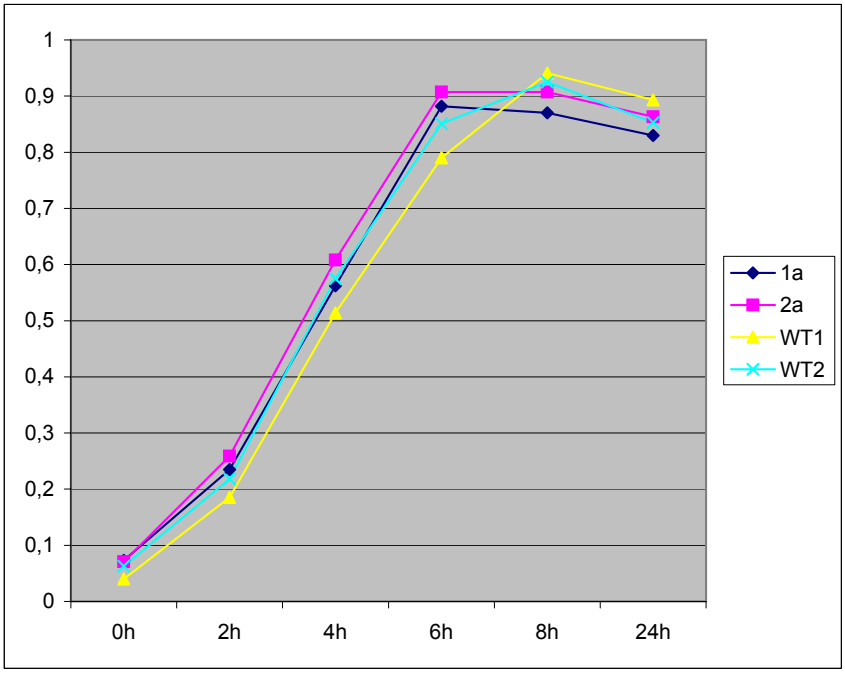


Fig.2
The growth curve for two wild types of Streptococcus intermedius compared with two luxS÷ mutants of S. intermedius. The growth curves, including the lag, log and the stationary phase were similar in the wild types and the mutants.

Biofilm formation in TSB

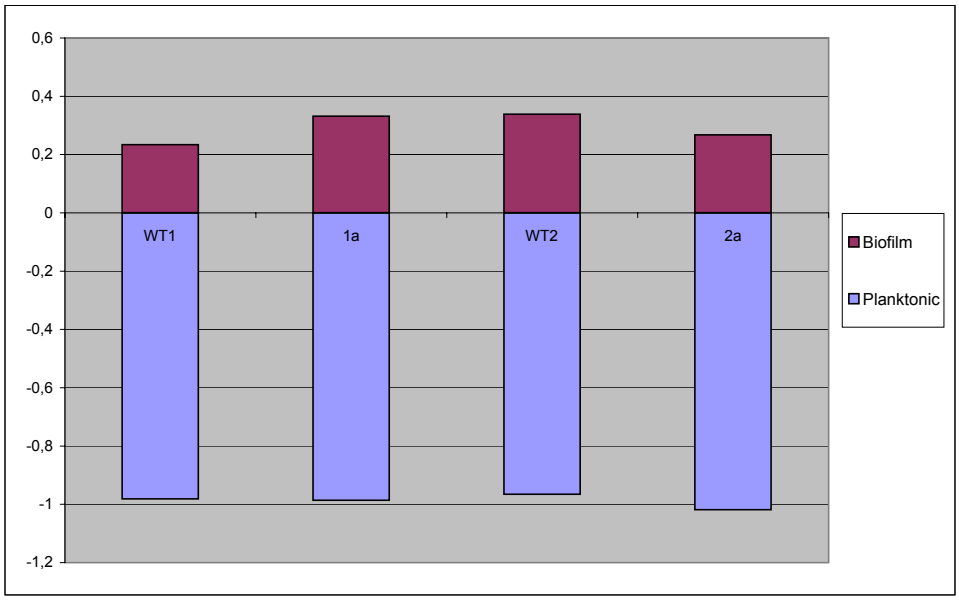


Fig. 3 Biofilm formation in TSB. The results from two independent experiments (1 and 2) are shown. The bars represent mean values from four parallels. Two different luxS isolates (1a and 2a) and the wild type (WT1 and WT2) were used in the experiments.

Mutant 1a formed slightly more biofilm than the wild type. This was in contrast to mutant 2a that formed less biofilm than the wild type. It was not possible in this pilot study to conclude whether inactivation of *luxS* has an effect on biofilm formation in TSB (fig 3). Further independent experiments allowing statistical analysis of the results are warranted before definite conclusions may be drawn. It is possible that the method could be refined to increase sensitivity.

Biofilm formation in BHI

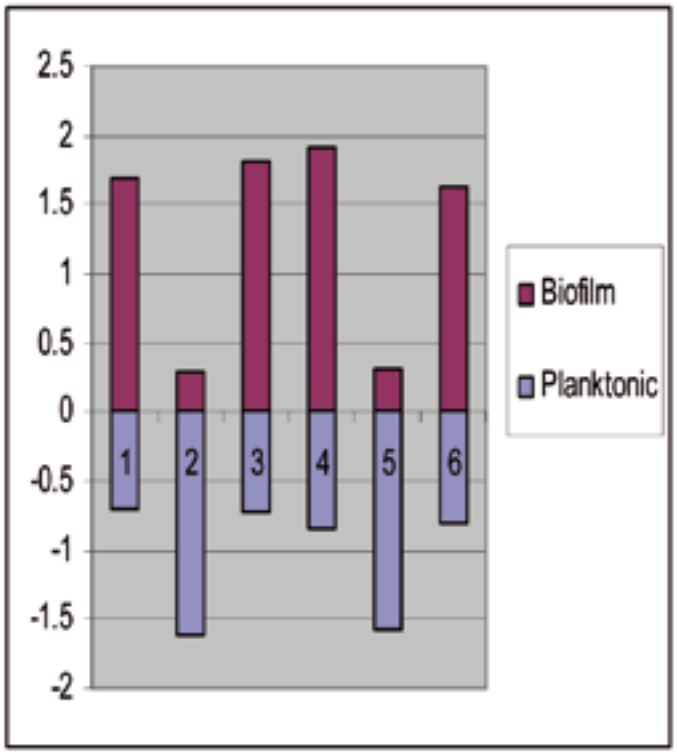


Fig.4 Average using BHI medium and different supplements. Diagrams of the wild type and mutant luxS÷, showing the amount of biofilm formed compared to planktonic bacteria. 1, 2 and 3 represent the Wild type and 4, 5 and 6 represent the mutant luxS÷. In diagrams 1 and 6 BHI and glucose was used. In diagrams 2 and 5 BHI was used and in diagrams 3 and 4 BHI and sucrose was used. The bars above 0 represent the OD values for biofilm, and below 0 the values for the planktonic fraction. The bars represent mean values of 3 independent experiments with 3 to four parallels.

When wild-types were subcultured on BHI medium, they showed no difference compared to the *luxS÷* mutants. The only remarkable difference was the increase in the production of biofilm, when adding glucose or sucrose. This increase was not significantly different when we compared the wild-type to the *luxS÷* mutants (fig.4).

Structural differences?

Biofilm formation in microtiter plates showed no difference in structure between the wild-type and the *luxS* deletion mutant.

We emphasized not only in obvious differences such as differences in shape or irregularity, but also in size and different ways of organization in chain formation.
Scanning electron microscopic images showed no apparent differences, except for the wild type grown in TSB alone, in which aggregates of cells were more often present (fig. 5).

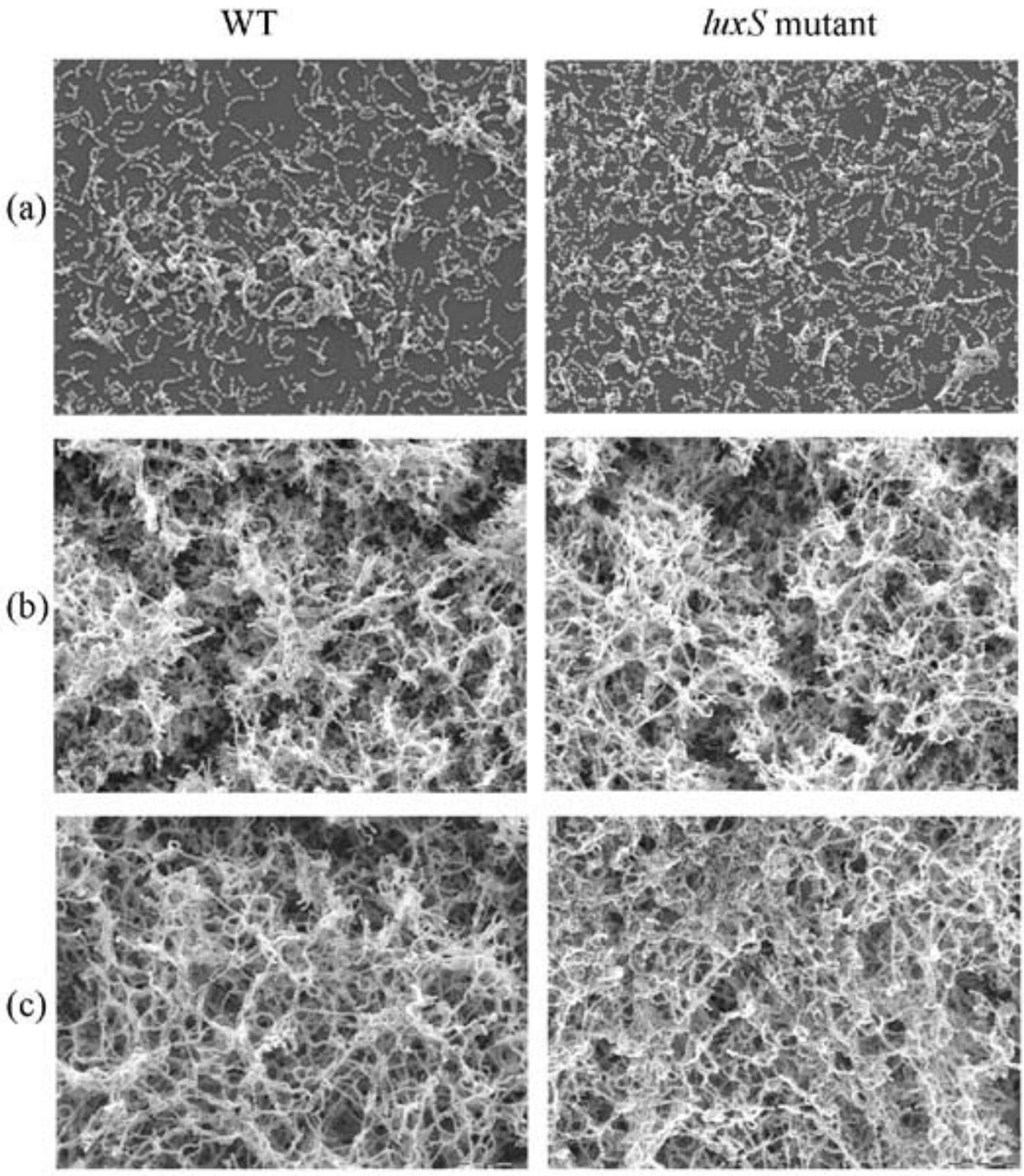


Fig. 5

Scanning electron microscopic images of the wild type compared with the luxS deletion mutant subcultured on: ***a)*** *BHI medium* ***b)*** *BHI supplemented with glucose* ***c)*** *BHI supplemented with sucrose.*

Discussion

As previously mentioned we wished to study whether the loss of the *luxs* gene was associated with changes in the biofilm formation and architecture. Given that this hypothesis was true one would expect a decrease in biofilm formation in the *luxS* deletion mutant, or at least a more irregular or looser architecture in the biofilm. The experiments were part of pilot studies, and more conclusive results might not be drawn. Refinement of the biofilm method and more experiments are warranted.
The possibility that *luxS* may be involved in *S. intermedius* biofilm formation under environmental conditions differing from those used in the present study might not be excluded. Such possibility is currently being examined as part of other ongoing projects, including conditions leading to more mature biofilms.

Conclusion

Not surprisingly are the subjects of quorum sensing and biofilm formation given a lot of attention. These efforts may actually result in the development of strategies for effectively controlling biofilm mediated infections.
A better understanding of how bacteria communicate in the formation of biofilms will also give us the knowledge of developing effective agents to intervene and to disconnect the bacterial communication, and hence prevent biofilm mediated infections.

The possibility to interfere with quorum sensing systems in oral bacteria may provide us a unique opportunity to prevent oral diseases in the future. Further research is needed to provide more information on the molecular mechanisms and signalling pathways in biofilms. This information will be of great clinical value in dentistry and medicine.

Acknowledgements

This study is part of a broader research, currently ongoing at the Department of oral biology, University of Oslo, by Anne Aamdal Scheie, Fernanda C. Petersen.
Thanks to Fernanda C. Petersen who constructed the *luxS* mutant and co-supervised this project. I would also like to thank Ali Oddin Naemi for laboratory advice and assistance and Steinar Stølen for providing the scanning electron microscopy pictures. Finally I would like to thank Anne Aamdal Scheie for giving me the opportunity to be a part of her research team and for supervising this project.

Literature cited

1. Zezhang T. Wen and Robert A.Burne. 2004. LuxS-mediated signalling in *streptococcus* Is Involved in Regulation of Acid and Oxidative Stress Tolerance and Biofilm Formation. Journal of Bacteriology 186.9.2682-2691(2004).

2. E .Peter Greenberg 2003. Bacterial communication: Tiny teamwork. Nature 424, 134 (2003).

3. Micheal J. Federle and Bonnie L. Bassler. 2003. Interspicies communication in bacteria. The journal of clinical Investigation 112.9. 1291-1299 (2003).

4. Marsh P, Martin M. Oral Microbiology. 4th ed. Oxford, (1999)

5. Carol Potera. 1999. Forging a Link Between Biofilms and Disease. Science 283.5409.1837-1839 (1999).

6. David G Davies, Matthew R. Parsek, James P Pearson, Barbara H. Iglewski, J. W. Costerton, E. P. Greenberg. The Involvement of Cell –to- Cell Signals in the Development of a Bacterial Biofilm. Science 280.295-297 (1998).

7. Justin Merritt, Fengxia Qi, Steven D. Goodman, Maxwell H. Anderson, and Wenyuan Shi. Mutation of *luxS* Affects Biofilm Formation in *Streptococcus mutans*. Infection and Immunity 71.4.1972-1979 (2003)

8. William R. Lyon, John C. Madden, James C. Levin, Jefferey L. Stein and Michael G. Caparon. Mutation of *luxS* affects growth and virulence factor expression in *Streptococcus pyogenes*. Molecular Microbiology 42.1.145-157 (2001)

9. David S. Blehert, Robert J. Palmer, Jr., Joao B. Xavier, Jonas S. Almeida, and Paul E. Kolenbrander. Autoinducer 2 Production by *Streptococcus gordonii* DL1 and the bifilm phenotype of a luxS Mutant Are Influenced by Nutritional Conditions. Journal of Bacteriology 185.16.4851-4860 (2003).

10. Rod McNab and Richard J. Lamont. Microbial dinner-party conversations: the role of LuxS in interspecies communication. Journal of Medical Microbiology 52.541-545 (2003).

11. Chicurel.M. Bacterial biofilms and infections. Slimebusters.Nature 408:284-6 (2000).

12. Yoshiaki, Kawamura Xiao-Gong Hou, Ferdousi Sultana, Hiroaki, Miura and Takayuki Ezaki. Determination of 16S r RNA sequences of *Streptococcus mitis* and *S. gordonii* and phylogenic relationships among members of the genus *Streptococcus*. International journal of systematic bacteriology 406-408 (1995).